Comparative Medicine
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Vol 57, No 3
June 2007
Pages 292–297

A Novel Swine Model for Evaluation of Potential Intravascular Hemostatic Agents

Harold G Klemcke,* Douglas S Cortez, Ashley B Cox, and Kathy L Ryan

Because uncontrolled hemorrhage is a leading cause of battlefield mortality, finding an intravenous treatment that could assist endogenous clotting mechanisms is a major mission for military researchers. Evaluation of potential intravenous hemostatic agents requires both in vitro and in vivo tests. For in vivo evaluation, we have developed a novel swine model in which 1) bleeding times (BT) and coagulation function could be ascertained after multiple doses of hemostatic drug administration and 2) a subsequent exsanguinating injury could be performed in the same animal, yielding screening information regarding the effects of drug pretreatment on blood loss and survival. Transection of small mesenteric arteries and veins allowed for multiple and reproducible BT measures that correlated with coagulation function. Subsequent excision of defined areas of the liver produced bleeding predominantly from small vessels (diameter, less than 2 mm) and parenchyma while resulting in 62% mortality without the use of either heparinization or aggressive fluid infusion. This swine model allows for multiple, repeatable BT measures in the same animal in experiments already involving laparotomy. Such a model is well suited for terminal studies to test effects of multiple doses of the same drug or multiple drugs on BT and allows for multiple, easily visualized measures that permit enhanced repeatability. The liver injury provides for numerous small vessel lesions that could be amenable to closure by coagulation. Therefore, drugs or mechanisms that enhance coagulation and concomitantly decrease blood loss and increase survival time may be accurately evaluated in this new model.

Abbreviations: BT, bleeding time; MAP, mean arterial pressure

Hemorrhage is the leading cause of death from wounds on the battlefield, accounting for over 50% of those deaths.[2,17] Hemorrhage is also the second leading cause of death in civilian trauma.[47] Approximately 80% of hemorrhagic combat deaths are from wounds that are not compressible (not accessible for manual pressure). Although the concept of pharmacologic hemostasis is not new,[13] currently there is no method available other than hospital-associated surgery that can effectively provide control for noncompressible hemorrhage. An intravenous drug that augments the body's innate clotting mechanisms, therefore, could provide an additional tool for hemorrhage control when surgical intervention is not possible. To evaluate the efficacy of such drugs, investigators traditionally have used in vivo models of bleeding time (BT) as well as more severe models of traumatic uncontrolled hemorrhage.

Although questions remain regarding its usefulness,[44] BT has been used as a test for diagnosis and prediction of potential bleeding abnormalities (especially of platelet disorders) and verification of therapeutic efficacy. Various procedures have been used for evaluation of BT in humans[43,44,51] and animals. Animal studies have used multiple species (mice, rats, rabbits, guinea pigs, dogs, and pigs) as well as multiple sites and methods of BT assessment (for example, tail, ear, buccal mucosa, spleen, skeletal muscle, skin, and mesentery).[1,6-12,20,21,31,35,36,39,40,42,45,56,58]

BT procedures used previously with pigs have heretofore consisted primarily of ear,[6,20,35,36,42,45] skin,[10,11,39] and splenic bleeds.[32] For each site, both shared and unique technical problems exist that limit the reproducibility, accuracy, and utility of BT measures. First, for all sites, length, depth, location and orientation of incision, and presence of occult blood vessels influence BT. Second, pigs' ears are often punctured, cut, and scarred as a result of swine husbandry practices, and interactions with pen-mates. This situation limits the usable ear surface area and hence the number of BT replicates that can be obtained. Third, maintaining a constant ear temperature is often problematic. To achieve this goal, some investigators place the ear into warm saline, but doing so results in greatly exaggerated bleeding times[6,35,36] and concomitantly prolongs the duration of studies. Fourth, good reproducibility and accuracy also depend upon skin temperature and thickness for skin bleeding. Finally, conduct of splenic BT[32] is exacerbated by the paucity of observations that can be made on the limited surface area. Further, the contractile nature of the pig spleen leads to altered splenic size and blood flow after manipulation.[14] Therefore, in an attempt to improve BT procedures previously used in swine, we developed an alternative approach using mesenteric vessels for experiments that already involve laparotomy.

Various animal models (rat, rabbit, dog, pig) also have been used for inducing a potentially treatable uncontrolled hemorrhage (hepatic, splenic, renal, and aortic injuries) within a body cavity.[17,22,24,26-28,34,38,50,55,57,59] Multiple models involving pigs have been used and include various liver injuries with[19,24,33,48,49] and without[25,29] various degrees of resuscitation. Hemodilution and hypothermia prior to liver injury also have been included in some models.[24,33,49] Finally, aortotomy with[4,52] and without[3] resuscitation and renal injury with[41] and without[16] hypothermia have been used in swine.

Each pig hemorrhage model has its assets. However, an in-

Received: 17 Apr 2006. Revision requested: 21 Aug 2006. Accepted: 24 Oct 2006.
US Army Institute of Surgical Research, Ft Sam Houston, TX.
**Corresponding author. Email: Harold.klemcke@amedd.army.mil*

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01 JUN 2007	**N/A**	**-**

4. TITLE AND SUBTITLE	5a. CONTRACT NUMBER
A novel swine model for evaluation of potential intravascular hemostatic agents.	5b. GRANT NUMBER
	5c. PROGRAM ELEMENT NUMBER
6. AUTHOR(S)	5d. PROJECT NUMBER
Klemcke H. G., Cortez D. S., Cox A., Ryan K. L.,	5e. TASK NUMBER
	5f. WORK UNIT NUMBER
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)	8. PERFORMING ORGANIZATION REPORT NUMBER
United States Army Institute of Surgical Research, JBSA Fort Sam houston, TX 78234	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)	10. SPONSOR/MONITOR'S ACRONYM(S)
	11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

15. SUBJECT TERMS

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**UU**	**6**	

herent deficit is that none appear to produce consistent results (that is, blood loss and mortality), a characteristic that could be detrimental for evaluation of potential intravascular hemostatic drugs. This deficiency may reflect the size of the injuries,[24] lack of repeatability in the number and size of blood vessels cut, or dilution of coagulation factors due to aggressive resuscitation.[52] Because of these issues, we sought to develop a new model using normothermic pigs with minimal preinjury hemodilution and no resuscitation that would be useful in screening intravascular agents for their ability to enhance coagulation function and reduce bleeding. We report herein an animal model in which 1) BT and in vitro hemostatic parameters can be measured multiple times and 2) a subsequent exsanguinating liver injury featuring primarily parenchymal and small-vessel bleeding can be created in the same animal.

Materials and Methods

Animals and instrumentation. Crossbred commercial swine weighing 38.4 ± 0.5 kg (mean ± standard error) were used in this study. A total of 27 pigs were used (3 for the heparin protamine test, 18 for the mesenteric BT evaluation, and 10 for the liver injury evaluation; 4 pigs were used in both the mesenteric bleed and liver injury evaluations). Animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. This study was approved by the Institutional Animal Care and Use Committee of the US Army Institute of Surgical Research (Fort Sam Houston, TX). Animals received humane care in accordance with the *Guide for the Care and Use of Laboratory Animals*.[37] Swine were fasted for 24 to 36 h before the procedure, with water continuously available. Animals were sedated using glycopyrrolate and tiletamine–zolazepam (Telazol, Fort Dodge, Overland Park, KS). Buprenorphine (0.9 mg intramuscularly) was administered for analgesia. They then were anesthetized (1% to 4% isoflurane in room air) and intubated, with use of a closed circuit system and mechanical ventilation. Infusion catheters were placed occlusively in a femoral vein and a jugular vein. Maintenance fluid (lactated Ringer solution; 5 ml/kg hourly) was infused continuously during the experiment until the liver injury phase (see following description).

An 8.5-French catheter introducer was shortened to 3 cm and placed occlusively in a femoral artery for blood sampling. A specialized catheter (Paratrend 7+ Multiparameter Sensor-catheter, Diametrics Medical, Roseville, MN) was placed occlusively into a carotid artery and was attached to an automatic blood gas monitoring system (Trendcare TCM 7000, Diametrics Medical) for continuous monitoring of body temperature and blood pH. A port in the catheter was coupled to a continuous data collection system (MicroMed, Louisville, KY) for monitoring blood pressure and heart rate. Laparotomy, splenectomy, and cystotomy were performed in each pig. To compensate for removal of the spleen, immediately after splenectomy each animal was infused with lactated Ringer solution at a volume equivalent to 3 times the spleen weight. Animals were stabilized for 10 min at a body temperature of 38.5 to 39.5 °C, a blood pH of 7.35 to 7.45, and a mean arterial pressure (MAP) that was at least 60 mm Hg.

Mesenteric BTs. After stabilization of the pig's condition, a distance of 30 cm was measured from the ileocecal junction along the ileum, and a second mark was made 15 cm further from this point. A U-shaped hollow plastic tube (diameter, 4 cm; length, 24 cm; filled with 39 °C saline) was laid under this 15-cm section of mesentery, and 3 small arteries with accompanying veins were identified within this area (Figure 1). These vessels were approximately 1 mm in diameter. Therefore, each group of 3 paired vessels (artery and vein) could be used to measure BT after a specific dose of a potential hemostatic agent. Each vessel pair was transected sharply with iris scissors, and the time to cessation of bleeding was measured, with 10 min (600 s) chosen (in light of preliminary observations) as the maximal BT possible; this observation period could, of course, be lengthened if treatments greatly prolonged BT. After transection, blood was collected with a 5-cm^2 gauze pad (The Kendall Company, Mansfield, MA) and cellulose sponge spears (Weck-Cel, Medtronic, Jacksonville, FL), which we touched very gently to the blood near the transected edges of the blood vessels; great care was taken to avoid touching the bleeding site directly. BT was then taken as an average of these triplicates at a given site on the mesentery, with elimination of a single value when the coefficient of variability (CV) exceeded 10%. BT was repeated along the mesentery twice more at 20-cm and 20-min intervals. Between BT measures, the pig's abdomen was covered with a towel soaked in 39 °C sterile saline, and the U-shaped tube was stored in an incubator maintained at 39 °C. Data from each set of replicate blood vessels at each of 3 sites along the mesentery (corresponding to 3 repeatable time intervals) from 18 different pigs subsequently were analyzed to determine the reproducibility of this procedure with respect to time after laparotomy and location within the mesentery relative to the ileocecal junction.

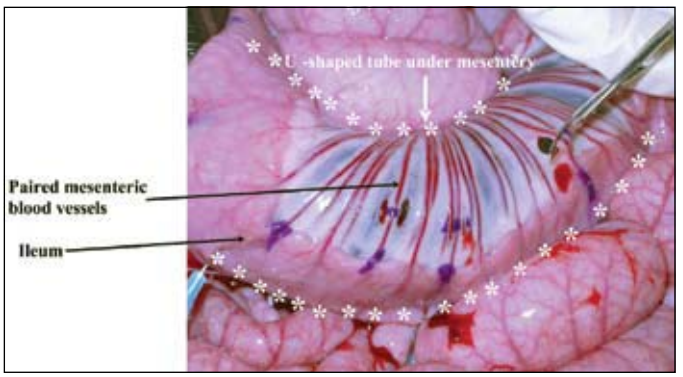

Figure 1. Photograph of pig small intestine with ongoing estimate of bleeding time that illustrates the paired mesenteric blood vessels. The outline of the U-shape tube underlying the mesentery is indicated with asterisks.

To determine the ability of BT measurements obtained with the described technique to reflect coagulation status, BT was measured in 3 additional pigs after infusion of heparin at 3 sequentially increasing doses (50, 75, and 100 IU/kg in 2 pigs, and 100, 200, and 300 IU/kg in 1 pig) and again after 3 administrations of 1 dose of protamine sulfate (0.5 mg/kg) to reverse the effects of heparin in the same 3 pigs. As described earlier, measures were made at different sites along the mesentery and at intervals of approximately 20 min.

Injury phase. After completion of BT determinations, a large uncontrolled hemorrhage was induced in 10 pigs. The peritoneal cavity was suctioned, and laparotomy sponges were positioned under the left medial and lateral liver lobes and within the gutters of the abdominal cavity. These sponges were clamped together for easy and immediate egress. Subsequently, the distances between the entry of the inferior vena cava into the liver and the caudal edges of both the left medial and left lateral liver lobes

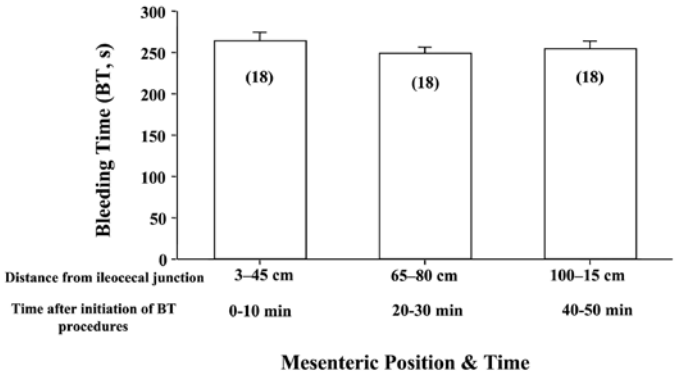

Figure 2. Bleeding time (BT) determination along the mesentery in 18 pigs. Each position corresponded to the indicated times after initiation of BT measures. Distances are measured along the small intestine from the ileocecal junction. Therefore, the first BT occurred at time 0 to 10 min at a distance of 30 cm from the ileocecal junction and in blood vessels that were present in the next 15 cm. After 10 min of observation and 10 min of rest, the second BT was performed 20 to 30 min after initiation of the first BT and at 65 cm from the ileocecal junction (20 cm from the previous BT zone). This process was repeated for a total of 3 BT, with BT performed in triplicate at each position. The average of these replicates became the BT measure for a given mesenteric position and time interval for each pig. Bars represent mean ± standard error of BT measured in 18 different pigs at each mesenteric location and time interval. Analysis of variance indicated an absence of differences ($P = 0.39$) in BT among mesenteric positions and time intervals.

were measured. Each lobe then was clamped loosely at approximately 45% of the distance from the caudal liver edge to the anterior liver edge adjacent to the entry of the inferior vena cava. A scalpel was used to cut these sections sharply to remove the distal aspects of each lobe. Therefore the percentage of the lobe cut was calculated as the length of the respective lobe removed divided by the overall length of that lobe and this result multiplied by 100%. The clamps then were removed, and the liver was allowed to bleed freely. All sponges were removed rapidly 30 s after excision, and the abdomen was closed temporarily. Infusion of maintenance fluid was discontinued, and no resuscitation fluids were provided. Animals were continuously monitored until death or for 2 h, at which point surviving animals were euthanized. After death, intraperitoneal blood was weighed via use of suction into preweighed canisters and gauze sponges; therefore, all blood loss measures are in grams. In addition, the number and size of transected vessels on the excised portions of the liver were measured and these data confirmed postmortem on the remaining liver sections. Vessels were classified as small (diameter, less than or equal to 2 mm), intermediate (diameter, 2 to 4 mm), or large (diameter, larger than 4 mm).

Blood sampling. Blood samples for activated clotting time (ACT) measures were collected concomitantly with BT measures at 20-min intervals by inserting a 20-cm single-use catheter made from vinyl tubing (inner diameter, 0.9 mm; Tygon, Saint Govaine Performance, Akron, OH) into the self-sealing port of the catheter introducer and gently withdrawing the femoral arterial blood to minimize shear-induced platelet activation. The first 3 ml of fluid removed was discarded to remove saline contained within the catheter and ensure that 100% blood was present in subsequent samples. ACT[15,30] was performed automatically (Hemochron Response, International Technidyne, Edison, NJ) according to the manufacturer's instructions.

Statistical analysis. Data were analyzed by use of the Statistical Analysis System software (SAS/STAT).[46] Bleeding times and ACT values were evaluated by analysis of variance (PROC GLM), in which pig was included as a random variable and means comparisons were made using either an a posteriori t test with the Hochberg adjustment for multiple comparisons, or where appropriate, the a priori procedure of orthogonal contrasts.[46] MAP values during BT procedures were analyzed by use of the PROC MIXED module with repeated measures. Correlation analyses were conducted by use of the PROC CORR module. Differences among numbers of cut blood vessels of various sizes were examined by use of PROC FREQ with the chi-square test and Hochberg adjustment for multiple comparisons. Where appropriate, data were tested for homogeneity of variance (PROC ANOVA with associated Levene test) and normality of distribution (PROC Univariate Normal with associated Kolmogorov–Smirnov test). Data were transformed where necessary to meet assumptions of analysis of variance. Data are presented as arithmetic mean ± standard error.

Results

BT determinations were made in 18 pigs. BT did not differ with section of mesentery ($P > 0.05$) nor with time during the bleeding procedure ($P > 0.05$; Figure 2). There was no relationship between MAP and BT when MAP was included in the statistical model as a covariate ($P = 0.49$). Indeed, when averaged across all pigs, MAP did not change during the 3 time intervals in which BT was measured (72 ± 3 versus 72 ± 3 versus 71 ± 3 mm Hg; $P > 0.52$).

To determine whether mesenteric BT reflected coagulation status, we measured BT in 3 pigs after heparin and subsequently after protamine administration. In vivo BT increased 106% ($P \leq 0.05$) with increasing doses of heparin (Figure 3; 0-H and 0-P versus D3-H and 0-P) and then decreased 56% after multiple administrations of protamine ($P \leq 0.05$; Figure 3). ACT varied in a similar manner (Figure 3), with 5.62-fold increases ($P = 0.05$) observed (0-H and 0-P versus D3-H and 0-P) and a 74% decrease ($P = 0.05$) subsequent to the last administration of protamine, compared with values for D3-H and 0-P. In vivo BT and ACT showed highly significant correlation ($r = 0.64$; $P = 0.003$).

After determination of BT, we performed a severe liver injury that resulted in death of 67% of the pigs during 2 h (Table 1). Approximately 41% and 48% of the left medial and left lateral lobes, respectively, were removed. Examination of these lobes demonstrated that 61% of the blood vessels cut were small vessels, and this percentage differed ($P < 0.01$) from those in the intermediate (23%) and large (16%) categories, which did not differ from each other ($P = 0.26$). There was no relationship between blood loss and survival time in these 10 pigs ($r = –0.1$; $P = 0.8$). Animal preparation, surgery, and mesenteric BT measures took approximately 2.5 h, as reflected in the duration of administration of maintenance fluid (Table 1). The volume of maintenance fluid provided represents approximately 17% of the pig's total blood volume, assuming 6.72 ml of blood per 100 g of body weight.[14]

Discussion

In this series of experiments, we were successful in developing a pig model in which intravascular hemostatic agents could be screened to determine their suitability for further testing for possible treatment of traumatic injury. This model allows the determination of hemostatic responses to escalating doses of drug,

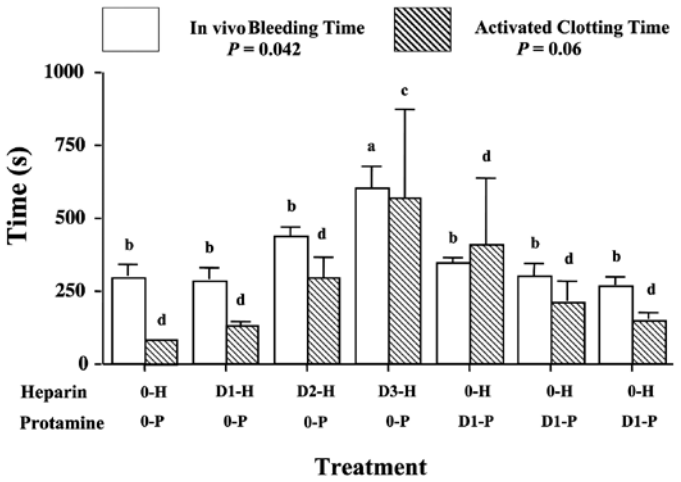

Figure 3. In vivo bleeding time (BT) and activated clotting time (ACT) after administration of heparin (doses 0-H, D1-H, D2-H, D3-H) and protamine (0-P, D1-P) in 3 pigs. Sequential administration of increasing doses of heparin occurred at approximately 20-min and approximately 20-cm intervals along the mesentery of the small intestine. Subsequently a single dose of protamine was administered at the same time and mesenteric intervals. Results of analysis of variance are given below the labels. Bars represent the means ± standard error. Means for BT and for ACT with common superscripts are not different ($P > 0.05$) as determined by a priori orthogonal contrasts or by t-tests with probability levels adjusted for multiple comparisons. For ACT, statistical analyses were conducted on $\log_{10}$-transformed data.

an approach that has been used previously at our institution.[42] In earlier studies, BT was determined by injury to either the ear[42] or spleen[32] of swine. Both methods are associated with technical problems, including the requirement to maintain a constant ear temperature, the inability to perform multiple BT measurements because of the limited surface area available for injury sites, the inability to perform more than 2 replicates at each BT measurement, and the contractile nature of the pig spleen. The use of mesenteric BT in an animal model that already involves laparotomy seems to obviate many of these problems and appears to be more reproducible than previous methods. Because of evident reproducibility between anatomical locations in the gut and with time, it is apparent that one can make BT determinations with multiple doses of hemostatic drug. In further support of this contention, blood flow is comparable among porcine duodenum, jejunum, and ileum.[60]

The primary blood supply to the porcine intestine is via the cranial mesenteric artery.[53] Within the mesenteries of the small intestine, the arteries and veins lie adjacent to each other, with a small amount of connective tissue between them.[53] Therefore, BT determination according to the current technique differs from other methods inasmuch as the mesenteric BT relies on bleeding from small arteries and veins rather than capillary bleeding. Despite this caveat, mesenteric BT clearly was sensitive to alterations in coagulation function because BT was correlated with alterations in ACT produced by infusion of heparin and protamine. Our model also enhances reproducibility; previous procedures involving supposed capillary bleeding routinely are confounded by cutting of a small unseen artery or vein.

Another feature of the pig model we developed is that it allows, within the same animal, preliminary determination of how pretreatment with a drug of interest might affect traumatic uncontrolled hemorrhage. In previous work, intravenous agents have been tested in a swine model (originally developed for testing the efficacy of hemostatic dressings) that included transection of major veins (diameter greater than 10 mm) of the liver.[24,33,42,48,49] Although entirely appropriate for testing hemostatic dressings, whether such a model is appropriate for testing intravenous hemostatic drugs is questionable, because such drugs likely would not be highly effective in such a situation (that is, big holes in big vessels). In addition, this previous model involved aggressive resuscitation that may not be entirely appropriate for testing intravascular agents because it leads to dilution of the intravascular agent under study as well as potential disruption of formed clots. Because of these considerations, we sought to develop a severe liver injury model that 1) was exsanguinating without either heparinization or aggressive resuscitation and 2) did not involve transection of the major veins of the liver but instead produced more diffuse bleeding from multiple small vessels. We also desired an injury that produced a mortality rate of approximately 50% in order to discern whether a drug under study had either beneficial or detrimental effects on survival. The selective excision of approximately 45% of both the left medial and lateral lobes described herein achieved these goals because it produced 62% mortality within 2 h through transection of predominantly small to intermediate (diameter, less than or equal to 4 mm) hepatic veins. Furthermore, this mortality rate was achieved without using either aggressive resuscitation or heparinization. In this study, we set fluid infusion at a low level that would defend against fluid loss from respiration, urination, and evaporation from exposed intestines that occurred during procedures. This infusion was stopped just prior to injury; no postinjury resuscitation was used. We therefore believe that this model is a reasonable one for testing of intravenous hemostatic agents.

Of interest, blood loss and survival time after liver injury lacked correlation. Ideally, models of uncontrolled hemorrhage would demonstrate positive correlation between these 2 variables. However, our results were not unexpected, because we

Table 1. Characteristics (mean ± standard error) of the pig liver injury model

Measure	
N	10
% Survival[a]	33
Survival time (min)[a]	81.1 ± 16.1
Blood loss (g)	860 ± 72
Blood loss (g/kg body weight)	21.3 ± 1.6
% Left medial lobe cut	41.4 ± 1.6
% Left lateral lobe cut	48.0 ± 3.5
No. (%) of small (≤2 mm) veins cut[b]	62 (61.4)
No. (%) of intermediate (>2 but ≤4 mm) veins cut[b]	23 (22.8)
No. (%) of large (> 4 mm) veins cut[b]	16 (15.8)
Maintenance volume (ml)	447 ± 35
Duration of maintenance fluid administration (min)	151 ± 15
Mean arterial pressure (mm Hg)[c]	65.9 ± 4.7
Heart rate (beats per min)[c]	166 ± 17
Blood pH[c]	7.41 ± .01

[a]n = 9; 1 pig euthanized at 1 h for nonexperimental reasons.
[b]Total number of veins cut was 101.
[c]Obtained just prior to liver injury.

have demonstrated in a rodent model of controlled hemorrhage that survival times differed widely among outbred rats with the same blood loss.[23] Such data suggest that differential survival time to hemorrhage is not entirely dependent on either volume or rate of blood loss but may also reflect contributions from the genetic and environmental background on which hemorrhage is overlaid. Because of this genetic and environmental contribution, uncontrolled hemorrhage models may be unable to achieve the desired correlation between blood loss and survival time.

In conclusion, we have developed a swine model for preliminary screening of intravenous hemostatic agents with potential efficacy in controlling traumatic bleeding. In this model, sequential blood sampling and determination of BT allows for collection of dose-response data for the test drug. Subsequent creation of liver injury within the same animal allows for preliminary screening of the test drug's applicability to exsanguinating hemorrhage. Importantly, the hepatic injury comprises injury predominantly to small vessels and the parenchyma, making our model more appropriate for testing of intravenous agents than previously used models.

Acknowledgments

The authors gratefully acknowledge the exceptional technical support of Jason Bliss, Michael Scherer, and Jacqueline Crissey as well as the outstanding staff of the Veterinary Service Support Branch at the US Army Institute of Surgical Research. This work was supported by funding from the US Army Medical Research and Material Command (Ft Detrick, MD).

The views expressed herein are the private views of the authors and are not to be construed as representing those of the Department of the Army or the Department of Defense.

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